MICROUTROPHIN AND USES THEREOF

STATEMENT OF FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

The present invention relates to the use of a microutrophin coding sequence in the treatment of muscular dystrophy.

Duchenne Muscular Dystrophy (DMD) is caused by a deficiency of the muscle cytoskeletal protein known as dystrophin. Dystrophin is a member of the spectrin superfamily of proteins and as such is distantly related to spectrin and alpha-actinin. Dystrophin is most closely related to the protein utrophin. The genes for these two proteins have nearly identical intron/exon structures, and the proteins are 50+% homologous at the amino acid level. Dystrophin is expressed throughout the entire length of the skeletal muscle fiber while utrophin is normally expressed only at the neuromuscular junction. Most cases of DMD result from sporadic deletions of the X chromosomal dystrophin gene. The destruction of the dystrophin open reading frame by these mutations suggests that therapies that genetically reconstitute dystrophin expression will elicit a cellular immune response against the fibers in which the protein is synthesized.

In the years following the initial discovery of utrophin, the technologies for targeted gene ablation in mice facilitated a formal genetic analysis of gene complementation. In the transgenic mouse in which the expression of utrophin is dictated by a muscle-specific promoter, utrophin can complement the physiological role of dystrophin.

Tinsley and Davies, US Patent No. 6,518,413, describe the expression of a polypeptide with utrophin function from a nucleic acid sequence for use in treatment of muscular dystrophy. This group designed a truncated protein modeled on a natural

mutation identified in a mild Becker muscular dystrophy patient. However, while the constructs provide some amelioration of symptoms, they are not optimal in terms of size, permissible delivery routes, or therapeutic outcome.

More recently, X. Xiao, US Patent Application Publn No. US 2003/0171312 A1 and J. Chamberlain, *et al*, US Patent Application Publn No. US 2003/0216332 A1, have described mini-dystrophin genes for use in treating muscular dystrophies. In the case of US 2003/0171312 A1, the dystrophin mini-gene may contain regions of the utrophin gene.

What is needed is an improved method of treating muscular dystrophies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A to 1N provide the sequences of a canine microutrophin cDNA of the invention [nucleotides 12-3497 of SEQ ID NO:1] in alignment with a human microutrophin coding sequences of the invention [SEQ ID NO: 6] and a mouse microutrophin coding sequence of the invention [SEQ ID NO: 7].

Figs. 2A to 2E provide the sequences of a canine microutrophin of the invention [SEQ ID NO:2] in alignment with a human microutrophin of the invention [SEQ ID NO: 4] and a mouse microutrophin of the invention [SEQ ID NO: 5].

Fig. 3A to 2K provide an alignment of the human utrophin protein [SEQ ID NO:3] and the human dystrophin protein [SEQ ID NO: 8]. The repeats and hinge regions are marked with respect to the utrophin protein above the sequence and for the dystrophin protein below the sequence.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a vector comprising a microutrophin cassette useful in a medicament for treatment of muscular disorders, including muscular dystrophy.

In another aspect, the invention provides a pharmaceutical composition comprising the vector comprising the microutrophin cassette.

In yet another aspect, the invention provides a method of treating muscular dystrophies using microutrophin.

In still another aspect, the invention provides the use of a vector comprising a microutrophin cassette in the preparation of a medicament for treatment of muscular dystrophies.

Still other aspects and advantages of the invention will be apparent from the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides microutrophin useful in treatment of muscle wasting disorders characterized by dystrophic pathology and symptoms. The severe muscle wasting disorders include Duchenne muscular dystrophy (DMD) and the less debilitating Becker muscular dystrophy. The invention further provides pharmaceutical compositions, medicaments, and methods of use thereof, for treatment of such disorders.

Without wishing to be bound by theory, the inventors believe that the present invention is advantageous over prior dystrophin-based therapies, because such therapies are anticipated to cause an autoimmune response in subjects lacking the ability to express a functional native dystrophin gene. Further, the inventors believe that the present invention is advantageous over the previously described utrophin-based constructs of Tinsley and Davies, due to its design and the improved methods for delivery described herein.

The term "muscle cell" or "tissue" refers to a cell or group of cells derived from muscle, including but not limited to cells and tissue derived from skeletal muscle, cardiac muscle, smooth muscle, e.g., from the digestive tract, urinary bladder and blood vessels. The constructs of the invention can be delivered in vitro or in vivo, depending upon the application. Thus, for example, an isolated cardiomyocyte would constitute a "muscle cell" for purposes of the present invention, as would a muscle cell as it exists in muscle tissue present in a subject. The term also encompasses both differentiated and nondifferentiated muscle cells, such as myocytes, myotubes, myoblasts, cardiomyocytes and cardiomyoblasts, and progenitor cells, for example, the muscle derived stem cells or the bone marrow derived stem cells that can become muscle cells after differentiation.

The "microutrophin" of the invention is a utrophin polypeptide having a functional portion of the "actinin-binding domain" of about 270 amino acids relative to the human utrophin which is located within the N-terminal utrophin region, at least functional portions of the proline-rich hinge regions 1 and 4 (H1) and (H4), and a portion of the C-terminal utrophin protein. The microutrophin contains internal deletions in the central rod repeat domains and a truncation in the C-terminal region downstream, but retains the proper phasing (*i.e.*, conformation) to retain the desired biological function of utrophin. This construct of the invention is described in detail below.

Utrophin shows substantial homology to dystrophin, with significant divergence occurring in the rod domain, where utrophin lacks repeats 15 and 19 and two hinge regions (*See e.g.*, Love *et al.*, *Nature* **339**:55 [1989]; Winder *et al.*, *FEBS Lett.*, **369**:27 [1995]). Human utrophin contains 22 spectrin-like repeats and two hinge regions. *See, e.g.*, Genbank® accession number X69086 and GenBank® accession number AL357149, which provides full-length human UTRN gene for utrophin and encoded protein. Homologs of utrophin have been identified in a variety of organisms, including mouse (Genbank® accession number Y12229), rat (Genbank® accession number AJ002967), and dog (GenBank® accession number NW-139836). The nucleic acid sequence of these or additional homologs can be compared to the nucleic acid sequence of human utrophin using any suitable methods.

The "microutrophin" polypeptide provided in SEQ ID NO:2 and described in the examples is an artificial polypeptide containing an internal deletion and a C-terminal deletion, with respect to the native utrophin polypeptide. More particularly, the microutrophin polypeptide of Fig. 2 contains the N-terminal region of utrophin, hinge 1 (H1), and hinge 2 (H2), an internal deletion from Repeat 4 through Repeat 21, and, Repeat 22 through the C-terminal region until about Exon 63. The C-terminal region from Exon 63 through the native C-terminal region is deleted. Thus, the N-terminal utrophin amino acids through hinge 2 (H2) are fused to amino acids of Repeat 22 through the C-terminal region of Exon 62. The coding sequences for this polypeptide are provided in SEQ ID NO:1.

However, the microutrophin of the invention is not limited to this precise construct. Desirably, a microutrophin polypeptide contains amino acids from the N-terminal region of utrophin, at least two of the hinge regions, and all or a portion of the C-terminal region. In one embodiment, the N-terminal region of utrophin comprises a polypeptide from the N-terminus to about the hinge region (e.g., about amino acid 1 to 268 based on the aligned human utrophin sequence in Fig. 3 [SEQ ID NO:3].); however, shorter or longer fragments of the utrophin sequence N-terminal to the hinge region may be selected. For example, 1 to 10, 1 to 5, 2, 3 or 4 of the first amino acids of the N-terminal sequences may be deleted. In one embodiment, the microutrophin is deleted of all or a fragment of hinge region 3. In another embodiment, the microutrophin is deleted of a fragment of hinge region 4. Suitably, the deletions are selected such that they permit proper conformational alignment of the utrophin protein, and particularly, retain the critical triple helices formed by the utrophin polypeptide. Preferably, the C-terminal cysteine-rich (CR) domain is truncated from a location at about Exon 63 [about amino acid 3346 of SEQ ID NO: 3] through the end of the utrophin protein. In another embodiment, a longer portion of the C-terminal region, e.g., about Exon 64 – end, about Exon 65 – end, about Exon 66-end, or more, can be retained. In one embodiment, the microutrophin comprises the N-terminal region of utrophin, at least hinges H1 and hinge 4 (H4) of utrophin gene, and at least four of the central rod repeats of the utrophin genes.

Preferably, for use in human subjects, human microutrophin sequences are selected in order to minimize any immune response. Similarly, for a dog, canine sequences are preferably selected. The appropriate locations of the N-terminal, C-terminal, and internal deletions described herein in the context of the human and canine sequences can be readily determined for other utrophin homologs, by preparing an alignment and comparison to the sequences of human utrophin using any suitable methods.

The sequences encoding the microutrophin polypeptide, or the fragments thereof which are fused in frame to generate the microutropin, can be obtained by conventional techniques. For the experiments described herein, the utrophin sequences were obtained by reverse transcriptase (RT) polymerase chain reaction

(PCR) techniques from tissue from a dystrophic animal. Alternatively, utrophin sequences may be obtained from other suitable sources, or suitable fragments may be prepared using synthetic methods. The source of the microutrophin sequences is not a limitation of the present invention.

The term "microutrophin gene" or "microutrophin coding sequences" refers to a nucleic acid molecule containing sequences encoding the microutrophin constructs described herein. These sequences may be those encoding the native utrophin fragments for the constructed microutrophin polypeptide. Alternatively, the microutrophin gene may contain a modified N-terminal domain in which DNA sequences surrounding the original protein translation initiation codon ATG are modified. The N-terminus of the microutrophin gene may be modified to improve expression efficiency without affecting the functionality of the gene product. For example, the original sequence surrounding the translation initiation ATG codon of the utrophin gene may be substituted by the Kozak sequence to increase the efficiency of protein synthesis. In one embodiment of the current invention, the three nucleotides upstream of the coding sequence may be changed from "AAA" to "CCA" and the fourth nucleotide in the coding sequence may be changed from "C" to "G". The modified sequences are useful to enhance the yield and/or purification of microutrophin protein synthesis.

The nucleic acid sequences encoding microutrophin can be generated using techniques known to those of skill in the art and engineered into an appropriate expression cassette under the control of regulatory sequences which direct its expression in a cell. Suitably, the microutrophin expression cassette is inserted into a vector for targeting to a desired host cell and/or into a subject. The term "expression cassette" refers to a construct of genetic material that contains coding sequences and enough regulatory information to direct proper transcription and translation of the coding sequences in a recipient cell.

The microutrophin expression cassette may be introduced into a mammalian subject using a variety of methods. It may be delivered as a naked DNA with or without hydrodynamic-based or electroporation-based procedures. The microutrophin expression cassette can also be delivered using a suitable vector. A gene transfer

"vector" refers to any agent, such as a plasmid, phage, transposon, cosmid, chromosome, liposome, DNA-viral conjugates, RNA/DNA oligonucleotides, virus, bacteria, etc., which is capable of transferring gene sequences into cells. Thus, the term includes cloning and expression vehicles, as well as non-viral and viral vectors.

Non-viral vectors such as liposomes or virus-liposome complexes, or with viral vectors such as adenovirus, HSV, baculovirus, retrovirus, lentivirus, and preferably AAV. Expression of the microutrophin minigenes may be controlled by a number of regulatory elements, including but not limited to, AAV inverted terminal repeat (ITR), retrovirus long terminal repeat (LTR), cytomeglovirus (CMV) immediate early promoter and/or enhancer, CMV enhancer and chicken β-actin promoter (CB promoter), α-actin promoter, myosin promoter, muscle-specific creatine kinase (MCK) promoter and/or enhancer, and the like. In one embodiment, the muscle-specific promoters, including modified versions of the above promoters and the synthetic muscle promoters, may also be used.

Optionally, a vector is targeted to specific cells by linking a target molecule to the vector. A targeting molecule is any agent that is specific for a cell or tissue type of interest, including for example, a ligand, antibody, sugar, receptor, or other binding molecule. The invention is also intended to include such other forms of vectors which serve equivalent functions and which become known in the art subsequently hereto. The term "transduction" denotes the delivery of a DNA molecule to a recipient cell either *in vivo* or *in vitro*, via a replication-defective viral vector, such as via a recombinant AAV virion.

As used herein the term "regulatory sequences" pertains to sequences operably linked to the encoded gene product. In addition to the major elements identified above, the macromolecular complex (*e.g.*, a vector) also includes conventional control elements that are operably linked to the transgene in a manner that permits its transcription, translation and/or expression in a cell transfected with the macromolecular complex.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are capable of effecting the

expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters that are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

In one embodiment, the regulatory sequences are optimized for expression in the muscle and/or comprise tissue-specific promoters. For instance, if expression in skeletal muscle is desired, a promoter active in muscle can be used. These include the promoters from genes encoding skeletal β-actin, myosin light chain 2A, dystrophin, muscle creatine kinase, as well as synthetic muscle promoters with activities higher than naturally-occurring promoters (see Li *et al.*, *Nat. Biotech.*, **17**:241-245 (1999)). However, one of skill in the art can readily select a suitable constitutive, inducible, or regulated promoter.

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart *et al*, *Cell*, **41**:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 promoter [Invitrogen]. Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of

commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied compounds, include, the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system [International Patent Publication No. WO 98/10088]; the ecdysone insect promoter [No *et al*, *Proc. Natl. Acad. Sci. USA*, **93**:3346-3351 (1996)], the tetracycline-repressible system [Gossen *et al*, *Proc. Natl. Acad. Sci. USA*, **89**:5547-5551 (1992)], the tetracycline-inducible system [Gossen *et al*, *Science*, **268**:1766-1769 (1995), see also Harvey *et al*, *Curr. Opin. Chem. Biol.*, **2**:512-518 (1998)], the RU486-inducible system [Wang *et al*, *Nat. Biotech.*, **15**:239-243 (1997) and Wang *et al*, *Gene Ther.*, **4**:432-441 (1997)] and the rapamycin-inducible system [Magari *et al*, *J. Clin. Invest.*, **100**:2865-2872 (1997)]. Other types of inducible promoters that may be useful in this context are those that are regulated by a specific physiological state, *e.g.*, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter for the transgene will be used. The native promoter may be preferred when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

Methods for assembling and producing a variety of different vectors defined herein are known to those of skill in the art and have been described in textbooks and in the literature. See, *e.g.*, Sambrook et al, Molecular cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (2000). Production of the vector is not a limitation of the present invention.

An "AAV vector" refers to vectors derived from an adeno-associated virus serotype, including human AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, avian

AAV, ovian AAV, etc., AAV7 [International Patent Application No. PCT/US02/33629], AAV8 [International Patent Application No. PCT/US02/33629], human AAV9 [International Patent Application No. PCT/US04/028817], among others which have been described [G. Gao, *et al.*, *J Virol.* 2004 Jun;**78**(12):6381-8; G. Gao, *et al*, *Proc Natl Acad Sci U S A*. 2003 May 13;**100**(10):6081-6. Epub 2003 Apr 25], and to vectors derived from more than one AAV serotype (hybrid AAV vectors). For example, a hybrid AAV vector may contain DNA sequences derived from both AAV-1 and AAV-2. An AAV vector can have one or more of the AAV wild-type genes deleted in whole or part, preferably the rep and/or cap genes, but retain functional flanking ITR sequences. AAV vectors can be constructed using recombinant techniques that are known in the art to include one or more heterologous nucleotide sequences flanked on both ends (5' and 3') with functional AAV ITRs. In the practice of the invention, an AAV vector can include at least one AAV ITR and a suitable promoter sequence positioned upstream of the heterologous nucleotide sequence and at least one AAV ITR positioned downstream of the heterologous sequence.

A "recombinant AAV vector plasmid" refers to one type of recombinant AAV vector wherein the vector comprises a plasmid. As with AAV vectors in general, 5' and 3' ITRs flank the selected heterologous nucleotide sequence. AAV vectors can also include transcription sequences such as polyadenylation sites, as well as selectable markers or reporter genes, enhancer sequences, and other control elements which allow for the induction of transcription. Such control elements are described more fully below. In addition, an "AAV vector" can be stably introduced into a cell line or cell lines for the purpose of viral particle production. Such a cell line is usually termed as AAV packaging cell line.

As used herein, the term "recombinant AAV", "recombinant AAV particle" or "recombinant AAV virion" is defined as an infectious, replication-defective virus composed of an AAV protein shell encapsidating (i.e., surrounding with a protein coat) a heterologous nucleotide sequence, which in turn is flanked 5' and 3' by AAV ITRs. In this regard, single-stranded AAV nucleic acid molecules (either the sense/coding strand or the antisense/anticoding strand as those terms are generally

defined) can be packaged into an AAV virion; both the sense and the antisense strands are equally infectious. When the recombinant AAV DNA is equal to or smaller than 50% of the full length viral genome (about 5,000 nucleotides), it can also be packaged as double-stranded hairpin-like DNA into AAV virion. Such virion is also fully infectious.

The term "recombinant AAV particle" or "recombinant AAV virion" also refers to a hybrid AAV particle in which the AAV protein shell and the encapsulated nucleotide sequence may be derived from AAVs of different serotype. For example, a hybrid AAV particle may contain AAV-1 capsid proteins and AAV-2 ITRs, or vice versa. It is also possible to create hybrid AAV capsid proteins using coding sequences from two or more AAV capsid genes. In addition, the capsid protein of a recombinant AAV may be manipulated by mutation, deletion, and/or insertion of amino acid sequence in order to modify the tropism of the recombinant AAV (Wu *et al.* J. Virol **74**, 8635-47 [2000]; Girod *et al.* Nat Med **5**, 1052-1056 [1999]).

A number of techniques for constructing recombinant AAV are known in the art. See, *e.g.*, U.S. Pat. No. 5,173,414, Lebkowski *et al.* Mol Cell Biol **8**, 3988-3996 [1988]; Carter B J, Current Opinion in Biotechnology **3**, 533-539 [1992]; Muzyczka N, cited supra; and Zhou et al. J. Exp. Med. 179, 1867-1875 [1994]; Xiao *et al.* J. Virol. **72**, 2224-32 [1998]; also, International Patent Appln No. PCT/US02/33629], AAV8 [International Patent Appln No. PCT/US02/33629], human AAV9 [International Patent Appln No. PCT/US04/028817], among others which have been described [G. Gao, *et al.*, *J Virol.* 2004 Jun;**78**(12):6381-8; G. Gao, *et al*, *Proc Natl Acad Sci U S A.* 2003 May 13;**100**(10):6081-6. Epub 2003 Apr 25].

Other suitable vectors may be selected for targeting to a desired host cell including, *e.g.*, adenovirus, retroviral, lentivirus, and plasmids. Suitable methods for constructing adenoviral [e.g., S. Roy, *et al.*, Virology, 2004 Jul 1;**324**(2):361-72; WO 03/046124], lentiviral [*e.g.*, WO 01/83730; WO 99/61598; R. Zuffery *et al*, J. Virol., **72** (12):9873-9880 (Dec 1998); H. Miyoshi *et al*, J Virol, **72**(10):8150-8157 (Oct 1998) and plasmid vectors [see, *e.g.*, J. Sambrook, *et al*, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, NY (2000)] have been described.

Any of the above-described vectors carrying the microutrophin expression cassette may be formulated for delivery to host cells or a subject according to published methods. The vector is mixed with a physiologically compatible carrier for administration to a human or non-human mammalian patient. Suitable carriers may be readily selected by one of skill in the art in view of the route(s) of delivery. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (e.g., phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present invention.

Optionally, the compositions of the invention may contain, in addition to the vector and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The vectors are administered to a subject in an effective amount. By "subject" is meant any mammal, including, without limitation, humans and nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered.

As used herein, the term "effective amount" refers to a level which brings about at least partially a desired therapeutic or prophylactic effect in a tissue targeted by the method of the present invention. The infection with an effective amount of the vector carrying genetic material of interest can then result in the modification of the cellular activities, e.g., a change in phenotype, in a tissue targeted by the method of the present invention.

Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the liver or lung, orally, intranasally,

intratracheally, by inhalation, intravenously, intramuscularly, intraocularly, subcutaneously, intradermally, or by other routes of administration. Currently, intravenous and oral delivery routes are most desirable. However, other routes and combinations of different routes may be used, as desired.

Preferably, the constructs of the invention utilize promoters that direct expression in both skeletal and cardiac muscle. Such promoters may be constitutive promoters, examples of which are provided below. Alternatively, muscle specific promoters may be utilized. In one embodiment, the invention involves delivery of a microutrophin under the control of regulatory sequences comprising a promoter specific for skeletal muscle. In another embodiment, the invention involves delivery of a microutrophin under the control of regulatory sequences comprising a promoter specific for cardiac muscle. In still another embodiment, the invention involves delivery of a mixture of microutrophin vectors, one specifically targeting skeletal muscle and another specifically targeting cardiac muscle expression.

In one embodiment, delivery is accomplished by the global mycocardial perfusion method described in International Patent Application No. PCT/US2004/030463. In another embodiment, delivery is accomplished by the gene transfer methods described in International Patent Application No. PCT/US2004/031322, filed September 24, 2004. Briefly, this method involves transferring a microutrophin of the invention to muscle cells by exsanguinating a region of the subject's microvasculature and delivering the complex to this region under high hydrostatic pressure using a configuration of perfusion cannulae and balloon as required to protect heart and lung to protein the organs during perfusion. A balloon catheter having a balloon that extends substantially the full length of the aorta or vessel that is inserted into the subject is provided for use in the systemic delivery of vector. In still another embodiment, the invention provides for delivery via a perfusion circuit and surgical method is provided for delivering a substance to a subject's heart *in situ* during cardiopulmonary bypass surgery. The perfusion circuit defines a path for re-circulating a solution containing a macromolecular complex through a coronary circulation circuit through a subject's heart during a surgical

procedure in which the substance is prevented from being delivered to the subject's other organs. [US Patent Appln No. 60/614,892.]

Dosages of the vector will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective human dosage of the vector is generally in the range of from about 1 ml to about 100 ml of solution containing concentrations of from about 1×10^7 to 1×10^{16} genomes or particles vector. The dosage will be adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the recombinant vector is employed. The levels of expression of the transgene can be monitored to determine the frequency of dosage resulting in vectors, preferably AAV vectors containing the minigene. Optionally, dosage regimens similar to those described for therapeutic purposes may be utilized for immunization using the compositions of the invention.

Optionally, therapy with microutrophin can be combined with other therapies.

Expression of the microutrophin minigene may be detected by immunofluorescent staining and immunoblotting (Western blotting). Microutrophin therapy may be monitored by measuring missing DAP complexes on the myofiber plasma membrane, including the sarcoglycan complex which is typically not found in untreated dystrophic muscle due to the primary deficiency of dystrophin. Alternatively, microutrophin therapy can be monitored by assessing that muscle is protected from pathological phenotypes.

In one aspect, the invention provides a kit for use by a clinician or other personnel. Typically, such a kit will contain a microutrophin vector of the invention and, optionally, instructions for reconstitution and/or delivery thereof. In another embodiment, the kit will contain the microutrophin vector in a physiologically compatible saline solution and, optionally, instructions for dilution, and performing a method as described herein.

The kit of the invention may also contain a balloon catheter to facilitate somatic gene transfer as described [International Patent Application No.

PCT/US2004/030463, or by the gene transfer methods described in International Patent Application No. PCT/US2004/031322, filed September 24, 2004], oxygen-transporting agent and/or at least one disposable element of an extracorporeal circulatory support and oxygenation system. For example, at least one disposable element can be an oxygenator having a hollow body, a liquid inlet in fluid communication with the interior of the body, a liquid outlet in fluid communication with the interior of the body, a gas inlet for providing gas to the interior of a gas chamber, at least one gas-permeable membrane separating the gas chamber from the interior of the body, and a gas outlet for permitting gas to exit from the gas chamber, whereby gas exchange is enabled between a fluid in the interior of the body and a gas in the gas chamber. The oxygenator may be constructed as described in US Patent No. 6,177,403, wherein the gas-permeable membrane comprises PTFE tubing extending within at least a portion of the tube, and wherein the gas chamber comprises the interior of the PTFE tubing.

The following examples are illustrative of the invention. However, it will be understood that the invention is not limited to the following specified embodiments, or the methods or techniques for production or expression described therein.

Example 1: Generation of Viral Vector containing Microutrophin Expression Cassette

To obtain the microutrophin, mRNA was extracted from frozen aliquot of canine muscle and reverse transcribed into cDNA using the RETROscript system (Ambion). The cDNA was used as template for PCR using primers for canine utrophin. The PCR products were analyzed on 1.2% agarose gel.

Two microutrophin fragments were made by PCR cloning using Taq polymerase (ROCHE) and canine cDNA as the template. The first fragment cDNA was amplified with the primers , 5' CCG CGG GTA CCA GGA TCC GTC GAC ATC GAT CCA CCA TGG CCA AGT ATG GAG AA (sense, SEQ ID NO: 9) and Hinge 2 (Sal), 5' GTC GAC AGG AAT CTG TCT CTT CTT TGG (antisense; SEQ ID NO: 10) . The second fragment used the primers, 3' Exon70 TTA AGG ATC

CTC GAG TTT TTC AAG TCT CTA AGT TGT CAC C, SEQ ID NO: 11; Rpt 24 (Sal) 5'-GTC GAC CTG GAG AAG CTC AGA GAC-3'; SEQ ID NO:12.

Two microutrophin fragments were then joined at a Sal I site to form the microutrophin cassette. PCR TOPO (Invitrogen) cloning vector according to manufacture's instruction.

The plasmid DNA was isolated and analyzed by restriction analysis to confirm the presence of the insert. The DNA was sequence to verify the presence of the gene. The microutrophin gene was isolated from the plasmid DNA (with ClaI and XhoI restriction sites) and cloned into an AAV vector plasmid containing a cytomegalovirus (CMV) promoter and the small poly (A) signal sequence to generate the viral vector AAV2/1-CMV microutrophin. The recombinant AAV serotype 2/1 was prepared by published methods [A. Auricchio et al, J Clin Invest. 110(40:499-504 (Aug 15 2002); W. Xiao et al, J Virol, 73:3994-4003 (1999); US Patent No. 6,759,237].

Example 2: Expression of Functional Microutrophin

The mdx mouse (Bulfield et al. Proc. Natl. Acad. Sci. USA 81, 1189-1192 [1984]) is an animal model of DMD [purchased from Jackson Laboratory]. The genetic lesion in the mdx dystrophin gene is a nonsense mutation at base 3185 of the mRNA that causes premature termination of translation within exon 23. This nonsense mutation precludes synthesis of a functional protein. The mdx mouse model was used to assess the histological and western blot appearance of recombinant canine microutrophin.

Briefly, AAV2/1-microutrophin was into the right quadricep muscle of the mdx mice (intramuscular injection) with $1x10^{12}$ GC particles of purified virus AAV microutrophin. Muscle samples were collected for examination at various time points (approximately 1 to 2 months) after vector injection.

Muscle cryosections were immunofluorescently stained with utrophin (N-terminus) mouse monoclonal antibody (Vector Labs) and donkey anti- mouse FITC (Jackson ImmunoResearch). Slides were examined with a Nikon microscope.

Protein expression was observed in the neuromuscular junctions and in low level staining of sarcolemma and vessel walls in mdx mice. Molecular weights are 133 kd for the microutrophin.

The construct will be further assessed in a German Short haired Pointer dog, because of its complete deletion of the dystrophin coding sequence (SJ Schatzberg, et al, Neuromuscul Disord. 1999 Jul;9(5):289-95.).

All documents and GenBank® citations identified herein are incorporated by reference. Numerous modifications to, and variations of, the specific embodiments described herein will be readily apparent to one of skill in the art. The appended claims are intended to be construed to include all such embodiments and equivalent variations.